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(54) Title: USE OF FVIIa OR FVIIa ₁ FOR THE TREATMENT OF ADVERSE CONDITIONS RELATED TO THE FVIIa MEDIATED INTRACELLULAR SIGNALLING PATHWAY			
(57) Abstract <p>A novel intracellular signalling activity of coagulation factor VII (FVII) in cells expressing tissue factor (TF) is described. The present invention relates to use of FVIIa or another TF agonist, or FVIIa₁ or another TF antagonist for the preparation of a medicament for modulation of FVIIa-induced activation of the MAPK signalling pathway in a patient. Moreover the present invention relates to a method of treatment, and a method of detecting the activity of compounds, in particular drug candidates, that interact with the FVIIa mediated intracellular signalling pathway.</p>			

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TITLE

Use of FVIIa or FVIIai for the treatment of adverse conditions related to the FVIIa mediated intracellular signalling pathway

5 FIELD OF INVENTION

A novel intracellular signalling activity of coagulation factor VII (FVII) in cells expressing tissue factor (TF) has been described. The present invention relates to use of FVIIa or another TF agonist, or FVIIai or another TF antagonist for the preparation of a medicament for modulation of FVIIa-induced activation of the MAPK signalling pathway in a patient. Moreover the present invention relates to a method of treatment, and a method of detecting the activity of compounds, in particular drug candidates, that interact with the FVIIa mediated intracellular signalling pathway.

15 BACKGROUND OF THE INVENTION

The extrinsic pathway of blood coagulation is initiated when FVIIa circulating in plasma binds to the integral-membrane protein, tissue factor (TF). The role of TF in blood coagulation has been extensively studied (Camerer, E., A. B. et al. Thromb. Res. 81: 1-41, (1996)). The involvement of FVIIa as a proteolytic enzyme in the blood coagulation cascade is believed to be confined to the extracellular leaflet of TF expressing cells. An intracellular activity of FVIIa was first implied when the sequence of TF showed homology to the cytokine/ interferon- or heamatopoietic receptor superfamily (Bassoon, J. F. Proc. Natl. Acad. Sci. USA 87: 6934-6938, (1990)). The subclass I of the heamatopoietic receptor family includes receptors for growth hormone, prolactin, interleukins 1 to 7, granulocyte- macrophage colony stimulating factors, erythropoitin and thrombopoitin. Subclass II includes TF and receptors for interferon α and β (Wells, J.A., and De Vos, A.M. Annu. Rev. Biomol. Struct. 22: 329-351, (1993)).

The resemblance of TF to this class of receptors was further substantiated with the appearance of the crystal structure (Harlos, K., D. M. A. et al. Nature 370: 662-666, (1994), Mueller, Y. A., M. H. et al. Biochemistry 33: 10864-10870 (1994)). Characteristic of this class of cytokine receptors that includes receptors for interferon β and γ and IL-10 (Mott, H. R. and Campbell, I. D. Curr. Opin. Struct. Biol. 5: 114-121, (1995)) is that their activation lead to rapid tyrosine phosphorylation of the receptors themselves, as well as a subset of intracellular proteins. Within minutes after the initial tyrosine phosphorylation an array of mitogen-

activated (Ser/Thr) kinases (MAPK) is activated (Whitmarsh, A. J. and Davis, R. J. J. Mol. Med. 74:589-607, (1996)). These kinases are arranged in several parallel signalling pathways (David, M. et al. Science 269, 1721 (1996); Current opin. immunol. 8, 402-11 (1996)). Thorough studies of the putative intracellular signalling capacity of FVIIa have shown that it induce mobilisation of intracellular free-calcium (Ca^{2+}) in the human bladder carcinoma cell line, J82, which constitutively express TF and in umbelical vein endothelial cells which were pre-treated with interleukin-1 to express TF (Rottingen, J.-A. et al. J. Biol. Chem. 270: 4650-4660, (1995)), but have failed to show any cytokine-like activation of intracellular tyrosine kinases (Camerer, E., et al. J. Biol. Chem. 271: 29034-29042, (1996)). In conclusion FVIIa is believed, in a TF dependent manner, to induce mobilisation of intracellular Ca^{2+} through activation of phospholipase C (Camerer, E., et al. J. Biol. Chem. 271: 29034-29042, (1996)). The mechanism by which FVIIa activates phospholipase c is not known, but Camerer et al. specifically ruled out tyrosine kinase activation.

SUMMARY OF THE INVENTION

The present invention relates to usage of FVII and/or FVIIa and/or another TF agonist and/or FVIIai and/or another TF antagonist in therapeutic treatment of pathological conditions that can be related to or treated by specific activation or inhibition of the FVIIa mediated intracellular signalling pathway.

In accordance with the present invention it has been shown that binding of FVIIa to its receptor TF induces activation of the mitogen-activated protein kinase (MAP kinase) pathway including phosphorylation of tyrosines in MAPK/Erk1. TF is known to play a pertinent role in the pathogenesis of a number of diseased states where regulatory interference at the intracellular level is believed to be beneficial.

Thus, diseased states which may be treated are pathological conditions such as mechanical injury of blood vessels, atherosclerosis, ischemia/reperfusion, bacterial infection, tumour deposition, or stimuli induced by "stress factors" such as cytokines, smoking, high blood pressure, high lipids- or glucose levels, advanced glycosylation end-products, and bacterial lipopolysaccharides.

LIST OF FIGURES

Figure 1 shows the effect of zinc ions on TF-stimulated factor VIIa activity is shown in the absence (control) and presence of 0.2 mM cystin dihydroxamate. Factor VIIa activity was measured with the chromogenic substrate S2288 (H-D-Ile-Pro-Arg-p-nitroanilide). The activity of 10 nM FVIIa in the presence of 50 nM TF₁₋₂₁₈ (Dr W. Kisiel, University of New Mexico, Albuquerque, NM) was measured in buffer containing 50 mM TrisCl pH 7.4 0.1 M NaCl, 1 mM CaCl₂, 0.05 % Tween 20 and 0.4 mM S2288. The activity was measured at room temperature as the change in absorbance at 405 nm.

Figures 2A and 2B show the activation of SRE reporter gene expression induced by FVIIa upon binding to human TF.

Figure 3 shows the stimulation of BHK-TF/KZ136 cells stimulated with FVIIa with and without prior treatment with the MEK1/2 inhibitor PD98059. PD98059 was maintained at 50 μ M throughout the experiment.

Figures 4A and 4B show the specific activation of the Elk1 transcriptional factor:

4A) Superimposition of the transmitted light image and the fluorescence image showing the transfection efficiency,

4B) LCPS obtained from BHK-TF cells transiently transfected with the two hybrid system with Gal4-Elk1 and gal4-luciferase.

Figures 5A and 5B show the activation of MAPK p44/42: BHK TF 103 #11-2 cell line; Lane 3-8: Stimulation with 100 nM FVIIa for the time period indicated.

Figures 6A and 6B show the activation of MAPK p44/42: ECV-304 cell line (ATCC CRL-1998); IL-1 β stimulated (Lane 2-5) and unstimulated (lane 7-9) cells exposed to 20 nM FVIIa for the time period indicated.

Figures 7A and 7B show the activation of MAPK p44/42: MDCK cell line (ATCC CCL-34) exposed to 10 nM FVIIa (Lane 1-5) and 10 nM FVIIai (Lane 6) for the time period indicated.

Figure 8 illustrates the competition experiment between FVIIa and FVIIai in BHK-TF/KZ136 cells.

Figures 9A and 9B show the transient activation of MAPK p44/42.

Figure 10 shows FVIIa-induced signalling via the MAPK pathway using truncated TF (TF lacking the C-terminal end).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of FVII or FVIIa or another TF agonist for the manufacture of a pharmaceutical composition for inducing or enhancing activation of the MAPK signalling pathway in a patient, in particular wherein phosphorylation of MAPK/Erk1/2 leads to activation of transcription factor Elk1.

The present invention also relates to the use of FVII, FVIIa or another TF agonist for the manufacture of a pharmaceutical composition for enhancing FVIIa-induced activation of the MAPK signalling pathway in a patient.

In a further aspect the present invention relates to the use of FVII, FVIIa or another TF agonist for the manufacture of a pharmaceutical composition for inducing or enhancing activation of transcription factor Elk1

In a still further aspect the present invention relates to the use of FVIIa or another TF antagonist for the manufacture of a pharmaceutical composition for inhibiting or preventing activation of the MAPK signalling pathway in a patient.

In a further aspect the present invention relates to the use of FVIIa or another TF antagonist for the manufacture of a pharmaceutical composition for inhibiting or preventing activation of transcription factor Elk1 in a patient.

In a still further aspect the present invention relates to the use of FVIIa or another TF antagonist for the manufacture of a pharmaceutical composition for inhibition or prevention of FVIIa-induced activation of the MAPK signalling pathway in a patient.

In an embodiment of the present invention it relates to the use of FVIIa or another TF agonist for the manufacture of a pharmaceutical composition for the treatment of re-endothelization, co-lateral revascularization in ischemia/reperfusion in myocardial infarction diabetic microangiopathy.

In another embodiment of the present invention it relates to the use of FVIIa or another TF antagonist for the manufacture of a pharmaceutical composition for the treatment of restenosis, cancer.

5 In a further aspect the present invention concerns a method for inducing or enhancing activation of the MAPK signalling pathway in a patient, which comprises administering an effective amount of FVII or FVIIa or another TF agonist to said patient. In one embodiment the activation leads to phosphorylation of MAPK/Erk1/2, which leads to activation of transcription factor Elk1.

10 In a still further aspect the present invention concerns a method for enhancing FVIIa-induced activation of the MAPK signalling pathway in a patient, which comprises administering an effective amount of FVII, FVIIa or another TF agonist to said patient.

In a further aspect the present invention concerns a method for inducing or enhancing activation of transcription factor Elk1 in a patient, which comprises administering an effective
15 amount of FVII or FVIIa or another TF agonist to said patient.

In a still further aspect the present invention concerns a method for inhibiting or preventing activation of the MAPK signalling pathway in a patient, which comprises administering an effective amount of FVIIa or another TF antagonist to said patient.

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In a further aspect the present invention concerns a method for inhibiting or preventing activation of transcription factor Elk1 in a patient, which comprises administering an effective amount of FVIIa or another TF antagonist to said patient.

25 In a still further aspect the present invention concerns a method for inhibiting or preventing FVIIa-induced activation of the MAPK signalling pathway in a patient, which comprises administering an effective amount of FVIIa or another TF antagonist to said patient.

In a particular embodiment the effective amount is a daily dosage from about 5 µg/kg/day to
30 about 500 µg/kg/day.

In a further embodiment the TF antagonist comprises a zinc-chelator which binds to FVIIa.

The present invention provides a mechanism for an intracellular activity of FVII and/or FVIIa that relates to stimulation of the MAPK signalling pathway. Such a mechanism provides the basis for establishing the involvement of FVII and/or FVIIa in pathological conditions in which TF expressing cells like endothelial cells, epithelial cells, fibroblasts, smooth muscle cells and monocytes/macrophages participate. The invention furthermore provides the basis for identifying specific pharmacological targets within the FVIIa mediated intracellular signalling pathway that are useful for therapeutic intervention.

Thus, the present invention relates to usage of FVII and/or FVIIa and/or FVIIai in therapeutic treatment of pathological conditions that can be related to or treated by specific activation or inhibition of the FVIIa mediated intracellular signalling pathway.

In accordance with the present invention it has been shown that binding of FVIIa to its receptor TF induces activation of the mitogen-activated protein kinase (MAP kinase) pathway including phosphorylation of tyrosines in MAPK/Erk1/2 leading to activation of transcription factor TFC/Elk1. TF is known to play a pertinent role in the pathogenesis of a number of diseased states where regulatory interference at the intracellular level is believed to be beneficial.

Modulation of FVIIa-induced signalling may be particularly useful at vascular sites where injury in its broadest sense leads to endothelial dysfunction. Such damage might include mechanical injury, atherosclerosis, ischemia/reperfusion, bacterial infection, tumour deposition, or stimuli induced by "stress factors" such as cytokines, smoking, high blood pressure, high lipids- or glucose levels, advanced glycosylation end-products, bacterial lipopolysaccharides e.t.c. All leading to vascular complications and endothelial dysfunction characterised at the cellular level by a complicated interplay between inflammatory cells, vascular cells and components of the coagulation system, the complement system and the fibrinolytic system. Leukocyte recruitment to such sites of dysfunctional endothelium is an important component of the host response to extravascular injury. At the location, release and surface expression of a number of leukocyte products serve to co-ordinate the inflammatory response. The local expression of TF on various cells, including monocytes, macrophages, fibroblasts, smooth muscle cells, endothelial cells and tumour cells is known to contribute significantly to the development of this response, and TF has been implicated as an important regulatory receptor in the development of various diseased states.

In another aspect, the present invention relates to a method of detecting drug candidates that modulate the FVIIa mediated intracellular signalling pathway, which method comprise

- a) culturing a TF expressing cell that contain a DNA sequence coding for a reporter gene
5 who's expression is regulated by a SRE promoter element
- b) measuring the expression of the reporter gene
- c) incubating the cell with a drug candidate, and
- d) measuring the expression of the reporter gene produced by the incubated cell and determining any change in the level of expression compared to the expression measured in
10 step b, such change being indicative of biologically active drug candidate in said cell, or the method comprise
- e) culturing a TF expressing cell
- f) measuring the level of protein phosphorylation of specific proteins in the FVIIa mediated intracellular signalling pathway
- 15 g) incubating the cell with a drug candidate, and
- h) measuring the level of protein phosphorylation of the specific protein produced by the incubated cell and determining any change in the level of protein phosphorylation compared to the level measured in step f, such change being indicative of a biologically active drug candidate in said cell, or the method comprise
- 20 i) culturing a TF expressing cell
- j) measuring the spatial localisation of a specific component of the FVIIa mediated intracellular signalling pathway that upon activation of the FVIIa mediated intracellular signalling pathway change intracellular localisation
- k) incubating the cell with a drug candidate, and
- 25 l) monitoring the localisation of the same component and detect any change in localisation compared to the location measured in step j, such change being indicative of a biologically active drug candidate in said cell.

Generally, the blood components which participate in what has been referred to as the
30 coagulation "cascade" are proenzymes or zymogens, enzymatically inactive proteins, which are converted to proteolytic enzymes by the action of an activator, itself an activated clotting factor. Coagulation factors that have undergone such a conversion and generally referred to as "active factors", and are designated by the addition of the letter "a" to the name of the coagulation factor (e.g. factor VIIa).

The term "zinc-chelator" is intended to comprise a compound that binds to factor VIIa and induces replacement of calcium ions with zinc ions within factor VIIa, thereby inhibiting the activity of factor VIIa or tissue factor-factor VIIa complex (TF-FVIIa).

5

A suitable TF antagonist according to the invention may be a zinc-chelating compound, e.g. a dihydroxamate or a dihydrazide with the hydroxamate or hydrazide groups located relative to each other in such a position that they are able to chelate a zinc ion. The zinc-chelating compound acts in combination with FVIIa. Zn^{2+} -ions exert their inhibitory action in

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competition with a stimulatory effect of Ca^{2+} -ions. It is predicted that Zn^{2+} -ions displace Ca^{2+} -ions from one or more calcium binding site(s) within FVIIa. Zinc-chelating compounds, e.g. hydroxamates and hydrazides, are capable of acting as powerful supporters for binding of zinc ions in competition with calcium ions. Specific compounds thereby potentiate zinc inhibition of the activity of the factor VIIa/tissue factor complex. The activity of factor VIIa in

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complex with tissue factor can be inhibited by a mechanism in which a zinc chelator binds to factor VIIa and facilitates replacement of Ca^{2+} with Zn^{2+} . By this action the chelator exerts a modulatory effect on TF at the normal concentration of free Ca^{2+} and Zn^{2+} ions in the blood.

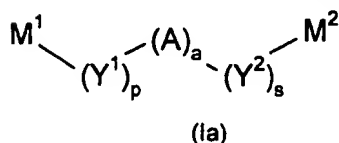
Demonstration that a suitable chelator potentiates zinc inhibition of factor VIIa/tissue factor activity.

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Figure 1 shows that the effect of zinc ions to abolish FVIIa-TF complex formation is profoundly potentiated by the zinc chelator, cystindihydroxamate.

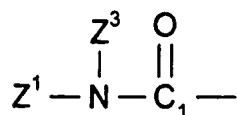
In one embodiment, the zinc-chelator is a compound of the general formula Ia

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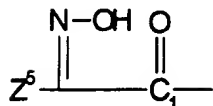


wherein

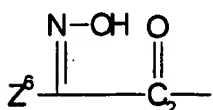
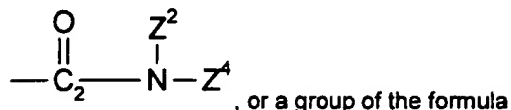
M^1 is heteroaryl, a group of the formula



, or a group of the formula



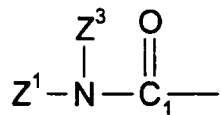
M² is heteroaryl, or a group of the formula



- 5 Z¹, Z², Z³ and Z⁴ independently of each other are hydrogen, C₁₋₄alkyl, hydroxy, amino or a valence bond attached to A,
 Z⁵ and Z⁶ represent a >C=O, which is attached to A,
 Y¹ and Y² independently of each other are a group of the formula -X¹~X²~X³-, wherein ~ independently of each other means a single or double bond, and X¹ represents >C=O, >CHR⁵,
 10 >CH₂, >CH- or a valence bond, wherein R⁵ is hydrogen, C₁₋₄alkyl, amino, C₁₋₄alkyl-amino, or di(C₁₋₄alkyl)amino, X² represents -NH-, >N-, >CH₂ or >CH-, and X³ represents -S-, >CH₂, >CH- or a valence bond,
 A is aryl or heteroaryl,
 p, a and s independently of each other are 0 or 1;
 15 or a pharmaceutically acceptable salt thereof;
 with the provisos that a+p+s is at least 1.

- In one embodiment of the above compound of general formula Ia, M¹ and M² are independently of each other pyridinyl, such as pyridin-2-yl. In a preferred embodiment only one of M¹
 20 and M² are pyridinyl, such as pyridin-2-yl.

In a second embodiment of the above compound of general formula Ia, M¹ is a group of the formula



- , wherein Z¹ and Z³ independently of each other are as defined above,
 25 or a group of the formula



In a third embodiment of the above compound of general formula Ia, M^2 is a group of the formula



10 In a further embodiment of the above compound of general formula Ia, the distance between C_1 and C_2 is from about 0.37nm to about 0.47nm.

In a still further embodiment of the above compound of general formula Ia, the distance between C_1 and C_2 is from about 0.47nm to about 0.57nm.

15 In a further embodiment of the above compound of general formula Ia, the distance between C_1 and C_2 is from about 0.57nm to about 0.67nm.

In a still further embodiment of the above compound of general formula Ia, the distance between C_1 and C_2 is from about 0.67nm to about 0.77nm.

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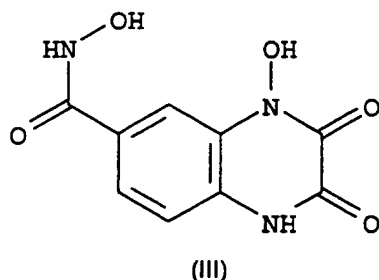
In a further embodiment of the above compound of general formula Ia, the distance between C_1 and C_2 is from about 0.37nm to about 0.77nm, preferably from about 0.40nm to about 0.70nm, more preferred from about 0.40nm to about 0.65nm.

25 In a still further embodiment of the above compound of general formula Ia, Z^1 , Z^2 , Z^3 and Z^4 are independently of each other hydrogen, methyl, hydroxy, amino or a valence bond attached to A. Preferably Z^1 , Z^2 , Z^3 and Z^4 are independently of each other hydrogen, hydroxy, amino or a valence bond attached to A.

- In a further embodiment of the above compound of general formula Ia, Y¹ and Y² are independently of each other a group of the formula -X¹~X²~X³-, wherein ~ independently of each other means a single or double bond, and X¹ represents >C=O, >CHR⁵, >CH₂, >CH- or a valence bond, wherein R⁵ is hydrogen, methyl, amino, methylamino, or di-methylamino, X² represents -NH-, >N-, >CH₂ or >CH-, and X³ represents -S-, >CH₂, >CH- or a valence bond. Preferably X¹ is >C=O, >CHR⁵ or a valence bond, wherein R⁵ is amino, X² is -NH- or >CH₂ and X³ is -S- or a valence bond.
- 10 In a still further embodiment of the above compound of general formula Ia, A is phenyl, 1,2,3-triazolyl, 1,2,4-triazolyl, or pyrazolyl.

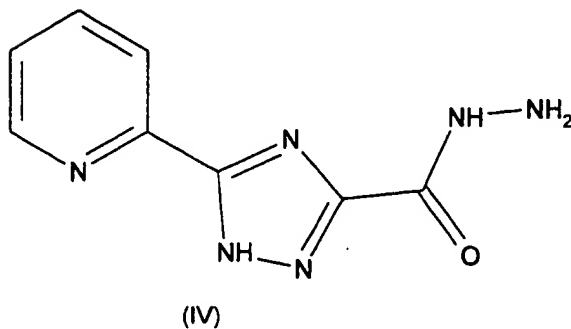
In a particular embodiment of the above compound of general formula Ia, the compound is selected from:

- 15 1-hydroxy-7-hydroxycarbamoylquinoxaline-2,3(1H,4H)-dione, having the formula III

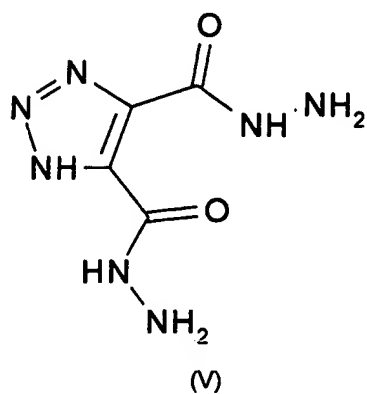


5-(2-pyridyl)-1,2,4-triazole-3-carbohydrazide, having the formula IV

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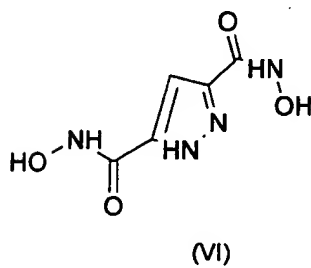


1,2,3-triazole-4,5-dicarbohydrazide, having the formula V



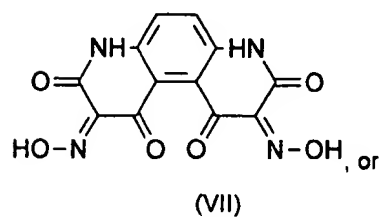
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Pyrazole-3,5-dicarbohydroxamic acid, having the formula VI



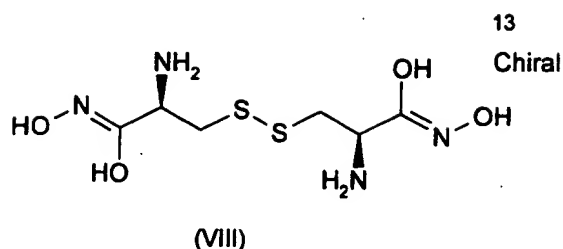
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4,7-Dihydro-[4,7]phenanthroline-1,2,3,8,9,10-hexaone-2,9-dioxime, having the formula VII



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L-Cystine dihydroxamate, having the formula VIII



5 Definitions

The term "FVII" means "single chain" coagulation factor VII

The term "Factor VIIa", or "FVIIa" means "two chain" activated coagulation factor VII cleaved by specific cleavage at the Arg152-Ile153 peptide bond. FVIIa, may be purified from blood or produced by recombinant means. It is evident that the practice of the methods described herein is independent of how the purified factor VIIa is derived and, therefore, the present invention is contemplated to cover use of any factor VIIa preparation suitable for use herein. Preferred are human FVIIa.

- 15 The term "FVIIai" is intended to mean FVIIa having at least one modification in its catalytic center, which modification substantially inhibits the ability of modified FVIIa to activate FX and FIX. Such modification includes amino acid substitution of one or more of the catalytic triad residues Ser344, Asp142 and His193, and also includes modification of catalytic triad residues with serine protease inhibitors such as organophosphor compounds, sulfanylfuoride, peptide halomethyl ketone or azapeptide. FFR ck FVIIa is one example of a FVIIai derivative obtained by blocking of the active center of FVIIa with the irreversible inhibitor, D-phenylalanine-L-phenylalanine-L-argininine chloromethyl ketone.
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The term "protein kinase" is intended to indicate an enzyme that is capable of phosphorylating serine and/or threonine and/or tyrosine in peptides and/or proteins.

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The term "MAPK signalling pathway" is intended to mean a cascade of intracellular events that mediate activation of Mitogen-Activated-Protein-Kinase (MAPK) and homologues thereof in response to various extracellular stimuli. Three distinct groups of MAP kinases have been identified in mammalian cells: 1) extracellular-regulated kinase (Erk), 2) c-Jun N-terminal kinase (JNK) and 3) p 38 kinase. The Erk MAP kinase pathway involves phospho-

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rylation of Erk 1 (p 44) and/or Erk 2 (p 42). Activated Erk MAP kinases translocate to the nucleus where they phosphorylate and activate transcription factors including (Elk 1) and signal transducers and activators of transcription (Stat).

- 5 The term "FVIIa-induced activation of the MAPK signalling pathway" is intended to indicate that FVIIa binds to TF in a mammalian cell and thereby induce activation of transcription factors Elk1 and Stat elements in a mammalian cell via phosphorylation of MAPK/Erk1/2.

- 10 The term "FVIIa mediated intracellular signalling pathway" is intended to indicate a cascade of intracellular events that involve activation of Erk1/2 MAPK.

- The term "drug candidate" is intended to indicate any sample which has a biological function or exerts a biological effect in a cellular system. The sample may be a sample of a biological material such as a microbial or plant extract, or it may be a sample containing a compound
15 or mixture of compounds prepared by organic synthesis or genetic techniques.

The term "TF agonist" comprises compounds inducing

- a) signal transduction by direct binding to TF (e.g. FVIIa),
b) stimulation of MAPK cascade,
20 c) abrogation of MAPK inhibition (e.g. PTPase inhibitors),
which agonists are drug candidates as defined above.

The term "TF antagonist" comprises

- a) reagents which compete with FVIIa for binding to TF without transmission, e.g. FVIIai,
25 b) reagents which bind to FVIIa and prevent binding to TF, e.g. Zn hydroxamate,
c) reagents which inhibit signal transduction by interfering with members of the MAPK cascade,
which antagonists are drug candidates as defined above.

- 30 The term "pharmacological targets" is intended to indicate a protein that can alter the activity of the FVIIa mediated intracellular signalling pathway.

The term "reporter gene" is intended to indicate a DNA construct that, when transcribed, produces a protein that can be detected.

The term "transcription factor TFC/Elk1" or "transcription factor Elk1" is intended to comprise Elk1 (also known as p62 ternary complex factor, TFC) is an Ets-related transcription factor that mediates growth factor stimulation of the c-fos promoter. Elk1 binds to DNA in part via interaction with Serum Response Factor. Elk1 is a bona fide Erk substrate. SAPKs phosphorylation of Elk1 may mediate transcriptional activation of the fos promoter in response to a variety of stresses.

The term "SRE promoter element" means a DNA sequence that binds transcription factors induced by components present in serum.

The term "TF expressing cell" mean any mammalian cell, that expresses TF.

The term "protein phosphorylation" is intended to indicate phosphorylation of serine and/or threonine and/or tyrosine in peptides and/or proteins.

Modulation of FVIIa-induced activation of the MAPK signalling pathway in a patient is defined as the capacity of FVIIa or another TF agonist, or FVIIai or another TF antagonist to 1) either increase or decrease ongoing, normal or abnormal, signal transduction, 2) initiate normal signal transduction, and 3) initiate abnormal signal transduction.

In this context, the term "treatment" is meant to include both prevention of an adverse condition, such as restenosis, and regulation of an already occurring condition, such as bacterial infection, with the purpose of inhibiting or minimising the condition. Prophylactic administration of FVIIa or another TF agonist, or FVIIai or another TF antagonist is thus included in the term "treatment".

In this context, the term "one unit" is defined as the amount of factor VII present in 1 ml of normal plasma, corresponding to about 0.5 µg protein. After activation 50 units correspond to about 1 µg protein.

In this context, the term "patient" is defined as any animal, in particular mammals, such as humans, suffering from a condition which may be treated by inhibition or activation of the MAPK signalling pathway.

Abbreviations

- TF tissue factor
FVII factor VII in its single-chain, unactivated form
5 FVIIa factor VII in its activated form
rFVIIa recombinant factor VII in its activated form
FVIIai modified factor VII

Pharmaceutical administration

- 10 The regimen for any patient to be treated with FVIIa or another TF agonist or FVIIai or another TF antagonist as mentioned herein should be determined by those skilled in the art. The daily dose to be administered in therapy can be determined by a physician and will depend on the particular compound employed, on the route of administration and on the weight and the condition of the patient. An effective amount is suitably a daily dosage from about 5 $\mu\text{g/kg/day}$ to
15 about 500 $\mu\text{g/kg/day}$, preferably from about 10 $\mu\text{g/kg/day}$ to 300 $\mu\text{g/kg/day}$, more preferred from about 15 $\mu\text{g/kg/day}$ to 200 $\mu\text{g/kg/day}$, most preferred from about 20 $\mu\text{g/kg/day}$ to 100 $\mu\text{g/kg/day}$.

- The FVIIa or another TF agonist or FVIIai or another TF antagonist should be administered in
20 one single dose, but it can also be given in multiple doses preferably with intervals of 4-6-12 hours depending on the dose given and the condition of the patient.

- The FVIIa or another TF agonist or FVIIai or another TF antagonist may be administered intravenously or it may be administered by continuous or pulsatile infusion. FVIIa or another TF
25 agonist or FVIIai or another TF antagonist is preferably administered by intravenous injections and in an amount of about 100-100,000 units per kg body weight, and preferably in an amount of about 250 - 25,000 units per kg body weight corresponding to about 5-500 $\mu\text{g/kg}$, a dose that may have to be repeated 2-4 times per 24 hours.

- 30 Pharmaceutical compositions

Conventional techniques for preparing pharmaceutical compositions which can be used according to the present invention are, for example, described in Remington's Pharmaceutical Sciences, 1985.

The compositions used according to this invention are prepared by methods known per se by the skilled art worker.

In short, pharmaceutical preparations suitable for use according to the present invention is

- 5 made by mixing FVII, FVIIa or another TF agonist or FVIIai or another TF antagonist, preferably in purified form, with suitable adjuvants and a suitable carrier or diluent. Suitable physiological acceptable carriers or diluents include sterile water and saline. Suitable adjuvants, in this regard, include calcium, proteins (e.g. albumins), or other inert peptides (e.g. glycylglycine) or amino acids (e.g. glycine, or histidine) to stabilise the purified factor VIIa.
- 10 Other physiological acceptable adjuvants are non-reducing sugars, polyalcohols (e.g. sorbitol, mannitol or glycerol), polysaccharides such as low molecular weight dextrans, detergents (e.g. polysorbate) and antioxidants (e.g. bisulfite and ascorbate). The adjuvants are generally present in a concentration of from 0.001 to 4% w/v. The pharmaceutical preparation may also contain protease inhibitors, e.g. apronitin, and preserving agents.

15

The preparations may be sterilised by, for example, filtration through a bacteria-retaining filter, by incorporating sterilising agents into the compositions, by irradiating the compositions, or by heating the compositions. They can also be manufactured in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile medium suitable for

20 injection prior to or immediately before use.

20

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof,

25 be material for realising the invention in diverse forms thereof.

25

Examples

Preparation of compound

- 30 Human purified factor VIIa suitable for use in the present invention is preferably made by DNA recombinant technology, e.g. as described by Hagen et al., Proc.Natl.Acad.Sci. USA 83: 2412-2416, 1986 or as described in European Patent No. 200.421 (ZymoGenetics). Factor VIIa produced by recombinant technology may be authentic factor VIIa or a more or less modified factor VIIa provided that such factor VIIa has substantially the same biological activity for blood

coagulation as authentic factor VIIa. Such modified factor VIIa may be produced by modifying the nucleic acid sequence encoding factor VII either by altering the amino acid codons or by removal of some of the amino acid codons in the nucleic acid encoding the natural FVII by known means, e.g. by site-specific mutagenesis.

5

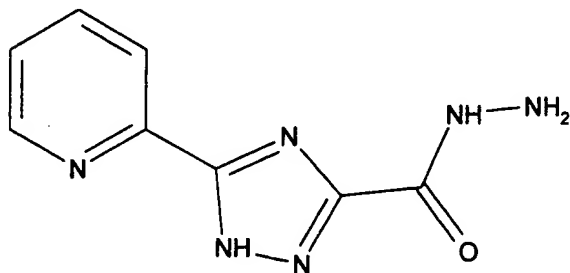
Factor VII may also be produced by the methods described by Broze and Majerus, J.Biol.Chem. 255 (4): 1242-1247, 1980 and Hedner and Kiesel, J.Clin.Invest. 71: 1836-1841, 1983. These methods yield factor VII without detectable amounts of other blood coagulation factors. An even further purified factor VII preparation may be obtained by including an additional gel filtration as the final purification step. Factor VII is then converted into activated FVIIa by known means, e.g. by several different plasma proteins, such as factor XIIIa, IX a or Xa. Alternatively, as described by Bjoem et al. (Research Disclosure, 269 September 1986, pp. 564-565), factor VII may be activated by passing it through an ion-exchange chromatography column, such as Mono Q® (Pharmacia fine Chemicals) or the like.

15

The following compounds are obtained from the indicated companies or universities:

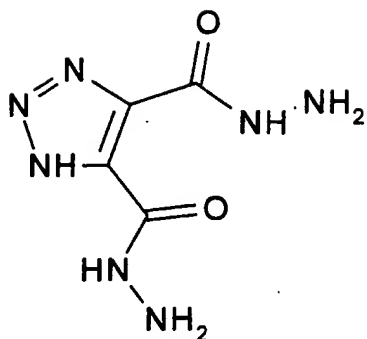
5-(2-pyridyl)-1,2,4-triazole-3-carbohydrazide (obtained from Maybridge Chemicals LTD (SEW 00446))

20



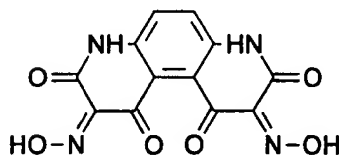
1,2,3-triazole-4,5-dicarbohydrazide (obtained from Odense University; is also disclosed in Farmaco, 50,(2) 1995, 99-106)

25



4,7-Dihydro-[4,7]phenanthroline-1,2,3,8,9,10-hexaone-2,9-dioxime (obtained from Labotest under the number (LT-2 AM36))

5



Example 1

10

Preparation of 1-Hydroxy-7-hydroxycarbamoylquinoxaline-2,3(1H,4H)-dione :

a) 4-Ethoxalylamino-3-nitrobenzoic acid

- 15 Anhydrous triethylamine (22.6 ml, 0.162 mol) was added to a solution of 4-amino-3-nitrobenzoic acid (14.4 g, 0.081 mol) in a mixture of dry tetrahydrofuran (300 ml) and dry N,N-dimethylformamide (100 ml). Then a solution of ethyl oxalylchloride (18 ml, 0.162 mol) in 100 ml of dry tetrahydrofuran was added dropwise at 0° C. The mixture was stirred overnight at room temperature and triethylamine hydrochloride was removed by filtration. The filtrate was evaporated to dryness and the residue was
- 20 triturated with water. The crude product was isolated by filtration and recrystallised from ethanol to give 14.4 g of the title compound which was used without further purification in the subsequent reductive cyclisation reaction. ¹H-NMR (DMSO-d₆): 1.35 (t, J = 7 Hz, 3H, CH₃), 4.36 (q, J = 7 Hz, 2H, CH₂), 8.2-8.6 (m, 3H, ArH), 11.6 (s, 1H, NH).

b) 7-Carboxy-1-hydroxyquinoxaline-2,3(1H,4H)-dione

A solution of 4-ethoxalylamino-3-nitrobenzoic acid (14.0 g, 49.6 mmol) in 800 ml of N,N-dimethylformamide was hydrogenated at room temperature and atmospheric pressure in the presence of 1.3 g of 5% platinum on carbon for 2.5 h. The catalyst was filtered off and washed with N,N-dimethylformamide. The filtrate was evaporated to dryness and the residue was triturated with 500 ml of water and filtered. The crude product was dissolved in 900 ml of 1M potassium dihydrogen phosphate buffer (pH 7.4), filtered and reprecipitated with 6 M hydrochloric acid to yield 7.7 g (70 %) of the title compound. ¹H-NMR (DMSO-d₆): 7.25 (d, J = 9 Hz, 1H, ArH), 7.75 (dd, J = 9 Hz, 2H, 1H, ArH), 7.98 (d, J = 2 Hz, 1H, ArH), 12.3 (br.s, 1H, exchangeable).

c) 1-Benzyloxy-7-carboxyquinoxaline-2,3(1H,4H)-dione

7-Carboxy-1-hydroxyquinoxaline-2,3(1H,4H)-dione (2.22 g, 10 mmol) was dissolved in a mixture of 50 ml of 1M potassium dihydrogen phosphate buffer (pH 7.4) and 25 ml of ethanol by gently heating. To the cooled mixture was added 1.19 ml (10 mmol) of benzylbromide and the mixture was stirred overnight at room temperature. The precipitate was isolated by filtration and washed with ethanol. The crude product was triturated with 4M hydrochloric acid and washed with water and dried *in vacuo* to give 1.56 g (50%) of the title compound. ¹H-NMR (DMSO-d₆): 5.22 (s, 2H, CH₂), 7.2-7.9 (m, 8H, ArH), 12.35 (s, 1H, exchangeable), 13.05 (br.s, 1H, exchangeable).

d) 1-Benzyloxy-7-(benzyloxycarbamoyl)quinoxaline-2,3(1H,4H)-dione

To an ice-cooled solution of 1-Benzyloxy-7-carboxyquinoxaline-2,3(1H,4H)-dione (422 mg, 1.35 mmol) in 10 ml of N,N-dimethylformamide was added 1-hydroxybenzotriazole (218 mg, 1.48 mmol) followed by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (272 mg, 1.42 mmol). Stirring was continued for 30 min at 0°C and O-benzylhydroxylamine hydrochloride (237 mg, 1.49 mmol) and dry triethylamine (0.21 ml, 1.5 mmol) was added. The mixture was stirred overnight at room temperature, then cooled and filtered. The isolated solid was successively washed with water, saturated aqueous sodium hydrogen carbonate and water. Recrystallisation from ethanol gave 290 mg (51 %) of the title compound. ¹H-NMR (DMSO-d₆): 4.95 (s, 2H, CH₂), 5.19 (s, 2H, CH₂), 7.2-7.8 (m, 13H, ArH), 11.8 (br.s, 1H, exchangeable), 12.3 (br.s, 1H, exchangeable).

e) 1-Hydroxy-7-hydroxycarbamoylquinoxaline-2,3(1H,4H)-dione

A suspension of 1-Benzyloxy-7-(benzyloxycarbamoyl)quinoxaline-2,3(1H,4H)-dione (250 mg, 0.6 mmol) in 50 ml of ethanol was hydrogenated at atmospheric pressure and room temperature for 1 h in the presence of 50 mg of 5 % palladium on carbon. Water (20 ml) and 4 ml of 2 N sodium hydroxide was added to dissolve the product and the catalyst was removed by filtration. The filtrate was acidified with 4 ml of 4M hydrochloric acid, evaporated to about 10 ml and filtered to give a white solid. Washing with a small amount of cold water and ethanol yielded 109 mg (70 %) of the title compound. ¹H-NMR (DMSO-d₆): 7.21 (d, J = 8Hz, 1H, ArH), 7.60 (dd, J = 8Hz, 2Hz, 1H, ArH), 7.90 (d, J = 2Hz, 1H, ArH), 9.05 (br.s, 1H, exchangeable), 11.35 (br.s, 1H, exchangeable), 11.82 (br.s, 1H, exchangeable), 12.35 (br.s, 1H, exchangeable).

Example 2

15 Synthesis of pyrazole-3,5-dicarboxylic acid (28-3028) on solid phase:

a) Synthesis of the linkage¹: a) Sasrin[®] resin (10.0 g, 0.73 mmol/g) was swelled in dichloromethane (40 mL) and diisopropylethylamine (40 mL), and cooled to 0°C. A solution of methanesulfonyl chloride (5.0 mL, 7.40 g, 64.6 mmol) in dichloromethane (20 mL) was added dropwise while stirring under argon, and stirring was continued for 30 min at 0°C and for 45 min at 25°C. Subsequently, the resin was drained and washed with dichloromethane (3 portions of 80 mL) and N-methylpyrrolidinone (NMP; 3 portions of 80 mL). b) In a 500 mL flask equipped with a mechanical stirrer, N-hydroxyphthalimide (23.8 g, 146 mmol) was dissolved in NMP (280 mL), and cesium carbonate (27.7 g, 73 mmol) was added. The mesylated resin was added in small portions at 25°C, and stirring was continued for 30 min at 25°C and for 16 h at 80°C. The chocolate-brown reaction mixture was poured into a Buchner funnel and washed extensively with methanol, water, methanol, dichloromethane, until the resin was colorless. c) The resin was suspended in ethanol (70 mL), anhydrous hydrazine (8 mL) was added, and the mixture was shaken at 25°C for 16 h. The resin was washed extensively with methanol, dichloromethane, methanol and dried; yield 9.50 g (95%).

b) Attachment of pyrrole-3,5-dicarboxylic acid to the resin prepared above: 0.10 g of the resin synthesized above was washed with N-methylpyrrolidone (1.5 mL). Subsequently, pyrrole-3,5-dicarboxylic acid (155 mg, 1.0 mmol), NMP (0.90 mL), 4-N,N-dimethylaminopyridine

(20 mg) in NMP (0.10 mL) and diisopropylcarbodiimide (78 μ L, 0.5 mmol) were added, and the mixture was shaken at r.t. for 120 min. Subsequently, the mixture was washed with NMP (4 portions of 2 mL).

- 5 c) A solution of PyBOP[®] (0.5 mmol, 260 mg) in NMP (250 μ L) was added to the resin. To this, a solution of hydroxylamine hydrochloride (70 mg, 1 mmol) in NMP (0.80 mL) / N-methylmorpholine (0.20 mL) was added, and the mixture was shaken at room temperature for 30 min. Subsequently, the resin was washed with dimethylformamide (3 portions of 2 mL) and dichloroethane (5 portions of 2 mL).

10

d) Cleavage: The resin was washed with dichloroethane (2 mL), and a mixture of 25% trifluoroacetic acid in dichloroethane (1.0 mL) was added. The mixture was shaken at r.t. for 15 min. The resin was filtered, the filtrate was collected, and the resin was washed with acetonitrile (2 portions of 0.80 mL). The solvents were evaporated in vacuo, and the crude sam-

15

ples were submitted to assay.

Lit.: L.S. Richter, M.C. Desai, *Tetrahedron Lett.* **1997**, *38*, 321.

EXAMPLE 3

20 Treatment of atherosclerosis:

Histochemical studies suggest that TF is a major determinant of the pro-thrombotic activity of human atherosclerotic lesions (Fernandez-Ortiz, A. et al. *J. Am. Coll. Cardiol.* 1562-1569, (1994)). Initiation of the coagulation cascade resulting in thrombin generation is important for fibrin deposition and the atherogenesis of the plaque. It is likely that also the cellular re-

25 sponse caused by TF-dependent signalling described in the present invention has important implications for atherogenesis and plaque development.

EXAMPLE 4

Treatment of angina and myocardial infarction:

- 30 TF is expressed on macrophages/foam cells associated with atherosclerotic plaques, and rupture of these structures are key events in the pathogenesis of unstable angina and myocardial infarction. It has been found that TF antigen concentration and activity in plaques from patients with unstable angina or myocardial infarction was significantly higher than in those from patients with stable angina (Ardissino, D. et al. *The Lancet* 349: 769-771, (1997)).

It is desirable to be able to interfere with and modulate the biological effects of TF expression to prevent a vicious spiral involving TF, whether this is caused by triggering of the coagulation system or it is a result of cellular signalling and consecutive reactions.

5 EXAMPLE 5

Treatment of cancer/angiogenesis:

TF is expressed on endothelial cells and tumour cells in breast cancer, but not on the same cells in benign fibrocystic breast disease (Contrino, J., *Nature Med.* 2: 209-215, (1996)). Local exposure of TF on the surface of specific cells in tumours appears to be crucial for vascularisation and growth of tumours (Folkman, J. *Nature Med.* 2: 167-168, 1996)). Recent studies have shown that blocking of a tumours blood supply with the angiogenesis inhibitors, angiostatin (O'Reilly, M. S. et al. *Cell* 79, 315-328(1994); Folkman, J. *Nature Med.* 1, 27-31(1995), WO9641194-A1) and endostatin (O'Reilly, M. S. et al. *Cell* 88; 277-285(1997)), or with antibody-directed targeting of TF (Huang, X. et al., *Science* 275: 547-550, (1997)) can arrest tumour or even cause tumour regression. Cellular signalling and orchestration of cellular transmitter substances is an important aspect of vascularization and tumour biology, and TF is likely to be of central importance in these processes. Reagents which modulate FVIIa-induced signalling is likely to work by a distinctly different mechanism and can provide an alternative to presently known angiogenic inhibitors.

20 EXAMPLE 6

Treatment of restenosis after clearing of blocked atherosclerotic vessels by surgical procedures.

Mechanical injury of the vessel wall results in local exposure of TF important to haemostasis and subsequent tissue repair. This is of immediate interest in relation to clearing of blocked atherosclerotic vessels by surgical procedures such as angioplasty, endarterectomy, reduction arterectomy or bypass grafting. These procedures result in serious vessel injury, TF exposure, thrombus formation and subsequent healing reactions. Proliferation of smooth muscle cells (SMCs) in the vessel wall is an important aspect of these events. The injury of the vessel is followed by medial SMC proliferation and migration into the intima, which characteristically occurs within the first few weeks and up to six month after injury and stops when the overlaying endothelial layer is established. In about 30% or more of patients treated by angioplasty, endarterectomy or bypass grafts, thrombosis and/or SMC proliferation in the intima causes re-occlusion of the vessel and consequent failure of the

reconstructive surgery. This closure of the vessel subsequent to surgery is known as restenosis. Modified factor VIIa (FVIIa) has been shown to effectively suppress the restenosis process (cf. WO 92/15686, title: modified FVII). This effect might be due to an inhibition of clot formation and thrombin generation initially after treatment of the constricted vessel. However, the present invention shows that in addition to being an antithrombotic drug, FVIIa is also an inhibitor of TF-dependent cellular signalling. Suppression of restenosis by FVIIa might therefore occur as a result of an effect on SMC proliferation or other cellular activities, and drugs which work as effectors of FVIIa-induced signalling could therefore represent a new and better strategy for the treatment of restenosis.

EXAMPLE 7

Reporter gene response: Activation of SRE reporter gene expression induced by FVIIa upon binding to the human TF.

BHK cells with and without stably transfected TF were stably transfected with KZ136 (reporter construct encoding 2x(STAT1,3), 2x(STAT4,5,6) and a serum response element (SRE) upstream to a luciferase reporter gene) were stimulated with FVIIa. Only cells expressing TF responded to FVIIa in a dose dependent manner. Fig. 2A shows that 20 nM FVIIa induced a response which was approximately two times higher than the background level. A maximal inducible FVIIa-response, three times higher than the background level, was reached at 100 nM FVIIa.

Fig. 2B shows that BHK cells not expressing TF did not respond to FVIIa addition. The responsiveness of the reporter system was controlled by addition of 15% FCS which showed a 3 times increase in luciferase activity over non-stimulated cells.

EXAMPLE 8

Monitoring the signalling pathway induced upon FVIIa - TF binding: The reporter gene approach.

A set of reporter vectors were stably transfected by selection into BHK cells already stably transfected with a construct driving expression of the TF. The constructs were KZ131, encoding one serum response element upstream to a luciferase reporter gene, KZ134, encod-

ing a cassette of two STAT1,3 elements and two STAT4,5,6 elements upstream to a luciferase reporter gene, KZ136, encoding a cassette of two STAT1,3 elements, two STAT4,5,6 elements and one serum response element upstream to a luciferase reporter gene, and KZ142, encoding the c-jun promoter upstream to a luciferase reporter gene.

5 Cells transfected with KZ131, KZ134 and KZ136 responded upon addition of FVIIa to the cells but cells transfected with KZ142 did not. Since the P44/42 MAPK pathway stimulates serum response elements and STAT elements these results indicate that TF upon binding of FVIIa activates the p44/42 MAPK pathway. The classical p44/42 MAPK do not activate the c-jun promoter.

EXAMPLE 9

Monitoring the signalling pathway induced upon FVIIa - TF binding: The MEK1/2 inhibitor approach.

15 PD98059 is a specific inhibitor of MEK1, a kinase specifically involved in the p44/p42 MAPK cascade. BHK cells stably transfected with TF and the KZ136 reporter construct pre-treated with 50 μ M PD98059 for one hour prior to stimulation with 100 nM FVIIa did not respond. Cells not pre-treated with PD98059 did respond well (Fig. 3) showing that TF is signalling
20 through the p44/42 MAPK pathway.

EXAMPLE 10

Monitoring the signalling pathway induced upon FVIIa - TF binding: The two hybrid approach.

In this approach the specific activation of the Elk1 transcriptional factor is monitored. BHK cells stably transfected with TF were co-transfected with the following vectors pFR-luc (20ug) (the reporter construct), pFA-Elk1 (0.5 μ g) (the Gal4-Elk1 chimera expression vector),
30 pFCdbd (14.4 μ g) (carrier DNA) and pEGFP-N1 (3 μ g) (reporter plasmid to monitor transfection efficiencies) (Clontech). pFRluc, pFA-Elk1 and pFCdbd are components of PathDetect system, Stratagene). A transfection efficiency of approximately 50% was estimated based on the number of cells expressing GFP (green fluorescent protein) (Fig. 4A). This mixture of transfected and non-transfected cells were stimulated with 100 nM FVIIa and assayed for

luciferase expression. Cells stimulated with 100 nM FVIIa showed a luciferase expression 5.1 times higher than the background level with a standard deviation of 3-5% (Fig. 4B) demonstrating that Elk1 is activated upon binding of FVIIa to cell-surface TF.

5 EXAMPLE 11

Monitoring the signalling pathway induced upon FVIIa - tissue factor binding: The antibody approach.

10 In this set of experiments with TF transfected BHK, ECV-304 and MDCK cell lines two antibodies raised against the MAPK were used, one targeting activated as well as non-activated forms of MAPK, and another, targeting only the activated (phosphorylated) form of MAPK.

BHK cells stable transfected with human TF were grown to 90% confluence and starved in
15 DMEM with 0.1% FCS for 24 hours prior to stimulation with FVIIa. Samples for Western blotting were sampled at 0, 3, 5, 7, 10, 20 and 40 minutes after addition of 100 nM FVIIa. The result is shown in Fig. 5A and B. The total amount of MAPK was essentially constant, whereas the antibody against the activated form of MAPK showed a temporally activation of MAPK p44/42 with a maximal activation at 3-7 minutes. Over the next 10 minutes the re-
20 sponse declined to reach the background level at about 20 minutes after addition of FVIIa.

Immortalised human endothelial cells (ECV-304) were grown to 90 % confluence and starved in medium 199 for 24 hours. In some experiments cells were exposed to IL-1 β for five hours to further increase expression of cell surface TF prior to addition of FVIIa (fig. 6A
25 and B).

Samples for Western blotting were taken at 0, 5 and 40 minutes after addition of 20 nM FVIIa to IL-1 β stimulated and unstimulated cells. The total amount of MAPK was essentially constant throughout the experiment whereas the activated form of MAPK showed a temporal
30 activation with a maximal activation at 5 minutes on both stimulated and unstimulated cells.

An epithelial Madin-Darby Canine Kidney (MDCK) cell line was grown to 100 % confluence and starved in DMEM for 48 hours prior to assay. Samples for Western blotting were drawn at 0, 5, 20 40 and 80 minutes after addition of 10 nM FVIIa. An additional sample stimulated

with 10 nM FVIIa was harvested after 40 minutes. Results shown in Fig. 7A and B. The total amount of MAPK was essentially constant whereas the activated form of MAPK showed a temporal activation with maximal phosphorylation at 20 minutes with a gradual decline at 40 and 80 minutes. No significant phosphorylation of MAPK was observed with FVIIa after 40 minutes exposure.

These examples show that FVIIa is capable of inducing phosphorylation of MAPK/Erk 1/2 in different TF expressing cell lines from various species. Furthermore FVIIa is not able to activate the same phosphorylation in MDCK cells.

EXAMPLE 12

Experiments on competition between FVIIa and FVIIa.

BHK cells stably transfected with the human TF and the reporter plasmid KZ136 were grown to 90% confluence, starved in DMEM with 0.1% FCS for 16 hours and then stimulated with FVIIa or FVIIa (Fig. 8). 100 nM FVIIa did not induce a serum response in contrast to 20 nM and 100 nM FVIIa which significantly increased the response. Also shown in Fig. 8 is an experiment where competition between FVIIa and FVIIa was studied. FVIIa was added to the cells 1 hour prior to stimulation with 20 nM FVIIa. The response induced by 20 nM FVIIa was inhibited 27%, 59%, 77%, and 91% by the addition of 20 nM, 50 nM, 100 nM, and 500 nM FVIIa, respectively. This showed that FVIIa could not induce signalling, and also that FVIIa could prevent FVIIa-induced signalling presumably by competing with FVII for a mutual binding site on TF.

EXAMPLE 13

Characterisation of the signalling pathway induced upon binding of FVIIa to tissue factor.

In this set of experiments with TF transfected BHK cell lines we used a phospho-specific antibody against phosphorylated (Thr202/Tyr204) p44/42 MAPK and an antibody targeting total p44/42 MAPK (New England Biolabs, Beverly, MA).

BHK cells stable transfected with human TF were grown to 90% confluence and starved in DMEM with 0.1% FCS for 24 hours prior to stimulation. In Fig 9A the cells were stimulated with 100 nM FVIIa for 0, 3, 5, 7, 10 and 40 minutes before the cells were lysed and samples for Western blotting were taken. The results in Fig. 9A (lower panel) show that the total amount of MAPK was essentially constant, whereas the results with the antibody against the activated form of MAPK (upper panel) showed a transient activation of MAPK p44/42 with a maximal activation at 3-7 minutes that declined over the next 40 minutes.

A similar experiment was performed with non-transfected BHK(-TF) cells. In these control cells the MAPK was not activated by FVIIa but a phosphorylated MAPK response was obtained with serum (results not shown).

The results shown in Fig 9B were obtained when BHK(+TF) cells were exposed to 100 nM FVII, FVIIa, FVIIai, [Ala344]FVII or FXa for 5 min. No effect on the total amount of p44/42 MAPK level was observed (lower panel) whereas a profound activation was seen with FVIIa, less so with FVII, and no significant activation was induced by FVIIai, [Ala344]FVII or FXa (upper panel). This strongly suggests that FVIIa activity was needed. Since FXa did not produce a significant increase in p44/42 phosphorylation, a putative FVIIa-mediated generation of FXa could not account for p44/42 MAPK activation with FVIIa. A brief EDTA wash supposed to remove possible trace amounts of vitamin K-dependent coagulation factors from the cell surface prior to FVIIa exposure was included in some experiments. This was without any reduction in MAPK phosphorylation, again supporting the notion that downstream coagulation reactions were not involved.

In conclusion this example shows that FVIIa/TF induces a transient phosphorylation of the p44/42 MAPK and that the catalytic centre activity of FVIIa is required for this FVIIa-induced phosphorylation. Furthermore we conclude that an indirect signalling pathway involving FVIIa-mediated activation of FX is unlikely.

EXAMPLE 14

The C-terminal tail of tissue factor is not required for FVIIa-induced signalling via the MAPK pathway.

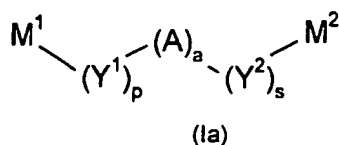
The cDNA coding for a truncated version of TF comprising the residues 1-247 was cloned into the mammalian Zem219b expression vector and transfected into BHK cells. This truncated TF without the C-terminal cytoplasmatic tail was expressed as a fully functional cofactor for FVIIa-

mediated FX activation. We used the phospho-specific antibody against phosphorylated (Thr202/Tyr204) p44/42 MAPK and an antibody targeting total p44/42 MAPK (New England Biolabs, Beverly, MA) to monitor the phosphorylation of MAPK.

- 5 BHK cells stable transfected with human TF(1-247) were grown to 90% confluence and starved in DMEM with 0.1% FCS for 24 hours prior to stimulation. The cells were then stimulated with 100 nM FVIIa for 10 minutes before the cells were lysed and samples for Western blotting were taken. The results is shown in Fig. 10. The total amount of MAPK was essentially constant (upper panel), whereas the activated form of MAPK of MAPK p44/42 (lower panel)
- 10 was phosphorylated as a result of exposure of the cells to FVIIa but not to FFR-FVIIa. In conclusion this example demonstrates that FVIIa/TF-induced signal transduction via the MAPK pathway takes place independent of the presence of the cytoplasmatic tail of TF.

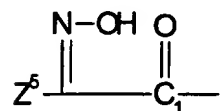
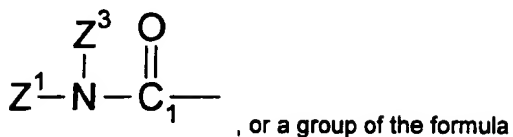
CLAIMS

1. The use of FVII or another TF agonist for the manufacture of a pharmaceutical composition for inducing or enhancing activation of the MAPK signalling pathway in a patient.
- 5 2. The use of FVIIa or another TF agonist for the manufacture of a pharmaceutical composition for inducing or enhancing activation of the MAPK signalling pathway in a patient.
3. The use according to claim 1 or 2 wherein said activation leads to activation of transcription factor Elk1 via phosphorylation of MAPkinase/Erk1/2.
- 10 4. The use of FVII, FVIIa or another TF agonist for the manufacture of a pharmaceutical composition for enhancing FVIIa-induced activation of the MAPK signalling pathway in a patient.
- 15 5. The use of FVII, FVIIa or another TF agonist for the manufacture of a pharmaceutical composition for inducing or enhancing activation of transcription factor Elk1 in a patient.
6. The use of FVIIai or another TF antagonist for the manufacture of a pharmaceutical composition for inhibiting or preventing activation of the MAPK signalling pathway in a patient.
- 20 7. The use of FVIIai or another TF antagonist for the manufacture of a pharmaceutical composition for inhibiting or preventing activation of transcription factor Elk1 in a patient.
8. The use of FVIIai or another TF antagonist for the manufacture of a pharmaceutical composition for inhibition or prevention of FVIIa-induced activation of the MAPK signalling pathway in a patient.
- 25 9. The use according to claim 6, 7 or 8 wherein said antagonist comprises a zinc-chelator.
- 30 10. The use according to claim 9 wherein said zinc-chelator is selected from a compound of the general formula Ia

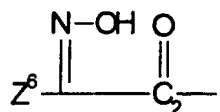
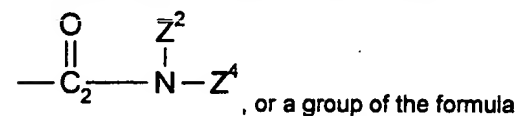


wherein

M¹ is heteroaryl, a group of the formula



5 M² is heteroaryl, or a group of the formula



Z¹, Z², Z³ and Z⁴ independently of each other are hydrogen, C₁₋₄alkyl, hydroxy, amino or a valence bond attached to A,

10 Z⁵ and Z⁶ represent a >C=O, which is attached to A,

Y¹ and Y² independently of each other are a group of the formula -X¹~X²~X³-, wherein ~ independently of each other means a single or double bond, and X¹ represents >C=O, >CHR⁵, >CH₂, >CH- or a valence bond, wherein R⁵ is hydrogen, C₁₋₄alkyl, amino, C₁₋₄alkyl-amino, or di(C₁₋₄alkyl)amino, X² represents -NH-, >N-, >CH₂ or >CH-, and X³ represents -S-, >CH₂,

15 >CH- or a valence bond,

A is aryl or heteroaryl,

p, a and s independently of each other are 0 or 1;

or a pharmaceutically acceptable salt thereof;

with the provisos that a+p+s is at least 1.

20

11. The use according to claim 9 or 10 wherein said zinc-chelator is selected from

1-hydroxy-7-hydroxycarbamoylquinoxaline-2,3(1H,4H)-dione,

5-(2-pyridyl)-1,2,4-triazole-3-carbohydrazide,

1,2,3-triazole-4,5-dicarbohydrazide,

25 Pyrazole-3,5-dicarbohydroxamic acid,

4,7-Dihydro-[4,7]phenanthroline-1,2,3,8,9,10-hexaone-2,9-dioxime, or

L-Cystine dihydroxamate.

12. The use according to any one of the preceeding claims wherein said patient is a mammal, such as a human.

5

13. The use of FVIIa or another TF agonist for the manufacture of a pharmaceutical composition for the treatment of re-endothelization, co-lateral revascularization in ischemia/reperfusion in myocardial infarction diabetic microangiopathy.

10 14. The use of FVIIa or another TF antagonist for the manufacture of a pharmaceutical composition for the treatment of restenosis or cancer.

15. A method of detecting a drug candidate that modulate the FVIIa mediated intracellular signalling pathway, the method comprising

- 15 a) culturing a TF expressing cell that contain a DNA sequence coding for a reporter gene, the expression of which is regulated by a SRE promoter element,
b) measuring the expression of the reporter gene,
c) incubating the cell with the drug candidate, and
d) measuring the expression of the reporter gene produced by the incubated cell and determining any change in the level of expression compared to the expression measured in step b, such change being indicative of the biologically active drug candidate in said cell.
- 20

16. A method of detecting a drug candidate that modulate the FVIIa mediated intracellular signalling pathway, the method comprising

- 25 a) culturing a TF expressing cell,
b) measuring the level of protein phosphorylation of specific proteins in the FVIIa mediated intracellular signalling pathway,
c) incubating the cell with the drug candidate, and
d) measuring the level of protein phosphorylation of the specific protein produced by the incubated cell and determining any change in the level of protein phosphorylation compared to the level measured in step b, such change being indicative of the biologically active drug candidate in said cell.
- 30

17. A method of detecting a drug candidate that modulate the FVIIa mediated intracellular signalling pathway, the method comprising

a) culturing a TF expressing cell,

b) measuring the spatial localisation of a specific component of the FVIIa mediated intracellular signalling pathway that upon activation of the FVIIa mediated intracellular signalling pathway change intracellular localisation,

c) incubating the cell with the drug candidate, and

d) monitoring the localisation of the same component and detect any change in localisation compared to the location measured in step b, such change being indicative of the biologically active drug candidate in said cell.

18. A method for assaying the ability of a drug candidate to modulate FVIIa-induced activation of the MAPK signalling pathway, comprising

a) culturing a TF expressing cell that contain a DNA sequence coding for a reporter gene, the expression of which is regulated by a SRE promoter element,

b) measuring the expression of the reporter gene,

c) incubating the cell with the drug candidate, and

d) measuring the expression of the reporter gene produced by the incubated cell and determining any change in the level of expression compared to the expression measured in step b, such change being indicative of the biologically active drug candidate in said cell.

19. A method for assaying the ability of a drug candidate to modulate FVIIa-induced activation of the MAPK signalling pathway, comprising

a) culturing a TF expressing cell,

b) measuring the level of protein phosphorylation of specific proteins in the FVIIa mediated intracellular signalling pathway,

c) incubating the cell with the drug candidate, and

d) measuring the level of protein phosphorylation of the specific protein produced by the incubated cell and determining any change in the level of protein phosphorylation compared to the level measured in step b, such change being indicative of the biologically active drug candidate in said cell.

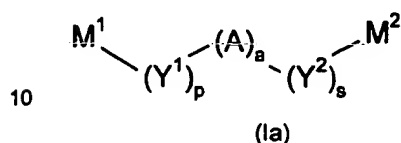
20. A method for assaying the ability of a drug candidate to modulate FVIIa-induced activation of the MAPK signalling pathway, comprising

- a) culturing a TF expressing cell,
 - b) measuring the spatial localisation of a specific component of the FVIIa mediated intracellular signalling pathway that upon activation of the FVIIa mediated intracellular signalling pathway change intracellular localisation,
 - 5 c) incubating the cell with the drug candidate, and
 - d) monitoring the localisation of the same component and detect any change in localisation compared to the location measured in step b, such change being indicative of the biologically active drug candidate in said cell.
- 10 21. A method for inducing or enhancing activation of the MAPK signalling pathway in a patient, which comprises administering an effective amount of FVII or FVIIa or another TF agonist to said patient.
22. The method according to claim 21 wherein said activation leads to activation of transcription factor Elk1 via phosphorylation of MAPK/Erk1/2.
- 15 23. A method for enhancing FVIIa-induced activation of the MAPK signalling pathway in a patient, which comprises administering an effective amount of FVII, FVIIa or another TF agonist to said patient.
- 20 24. A method for inducing or enhancing activation of transcription factor Elk1 in a patient, which comprises administering an effective amount of FVII, FVIIa or another TF agonist to said patient.
- 25 25. A method for inhibiting or preventing activation of the MAPK signalling pathway in a patient, which comprises administering an effective amount of FVIIai or another TF antagonist to said patient.
26. A method for inhibiting or preventing activation of transcription factor Elk1 in a patient,
- 30 which comprises administering an effective amount of FVIIai or another TF antagonist to said patient.

27. A method for inhibiting or preventing FVIIa-induced activation of the MAPK signalling pathway in a patient, which comprises administering an effective amount of FVIIa or another TF antagonist to said patient.

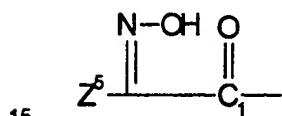
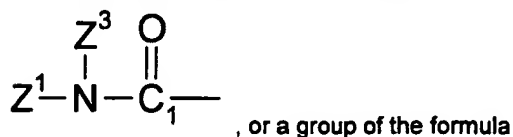
28. The method according to claim 25, 26 or 27 wherein said antagonist comprises a zinc-chelator.

29. The method according to claim 28 wherein said zinc-chelator is selected from a compound of the general formula Ia

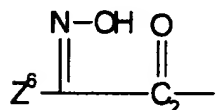
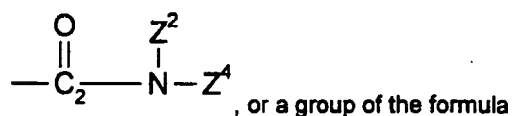


wherein

M¹ is heteroaryl, a group of the formula



M² is heteroaryl, or a group of the formula



20 Z¹, Z², Z³ and Z⁴ independently of each other are hydrogen, C₁₋₄alkyl, hydroxy, amino or a valence bond attached to A,

Z⁵ and Z⁶ represent a >C=O, which is attached to A,

Y¹ and Y² independently of each other are a group of the formula -X¹~X²~X³-, wherein ~ independently of each other means a single or double bond, and X¹ represents >C=O, >CHR⁵, >CH₂, >CH- or a valence bond, wherein R⁵ is hydrogen, C₁₋₄alkyl, amino, C₁₋₄alkyl-amino, or

di(C₁₋₄alkyl)amino, X² represents -NH-, >N-, >CH₂ or >CH-, and X³ represents -S-, >CH₂, >CH- or a valence bond,

A is aryl or heteroaryl,

p, a and s independently of each other are 0 or 1;

5 or a pharmaceutically acceptable salt thereof;

with the provisos that a+p+s is at least 1.

30. The method according to claim 28 or 29 wherein said zinc-chelator is selected from
1-hydroxy-7-hydroxycarbamoylquinoxaline-2,3(1H,4H)-dione,

10 5-(2-pyridyl)-1,2,4-triazole-3-carbohydrazide,

1,2,3-triazole-4,5-dicarbohydrazide,

Pyrazole-3,5-dicarbohydroxamic acid,

4,7-Dihydro-[4,7]phenanthroline-1,2,3,8,9,10-hexaone-2,9-dioxime, or

L-Cystine dihydroxamate.

15

31. The method according to any one of the preceeding claims wherein said patient is a mammal, such as a human.

32. The method according to any one of claims 21-31, wherein said effective amount is a
20 daily dosage from about 5 µg/kg/day to about 500 µg/kg/day.

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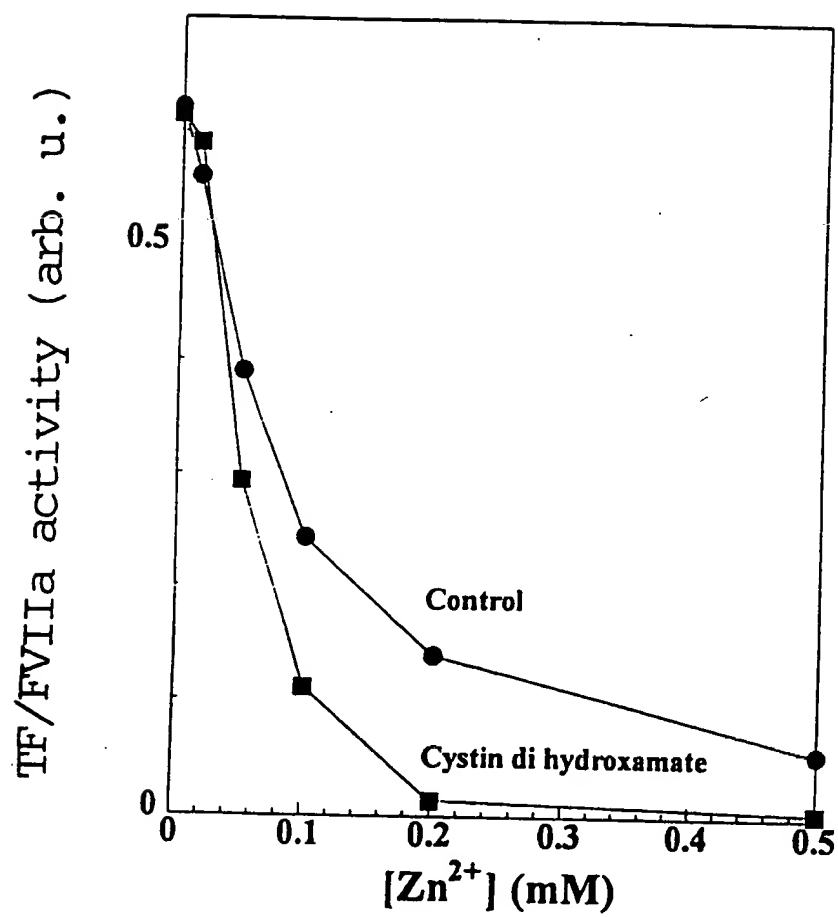


Fig. 1

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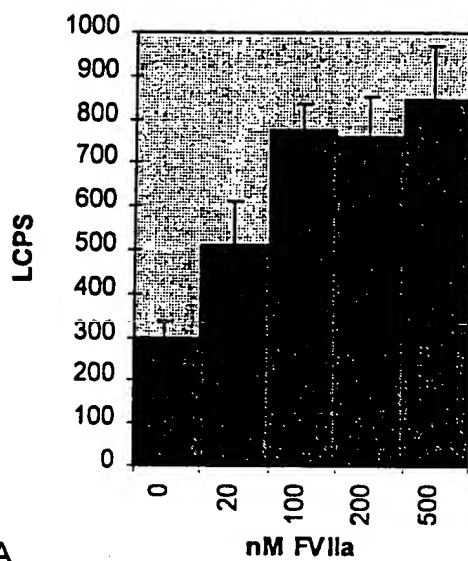


Fig. 2A

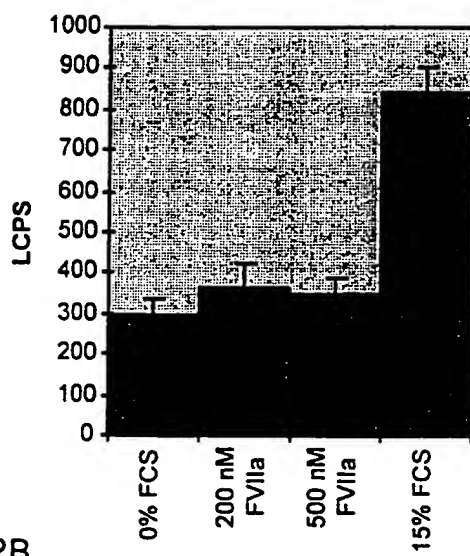


Fig. 2B

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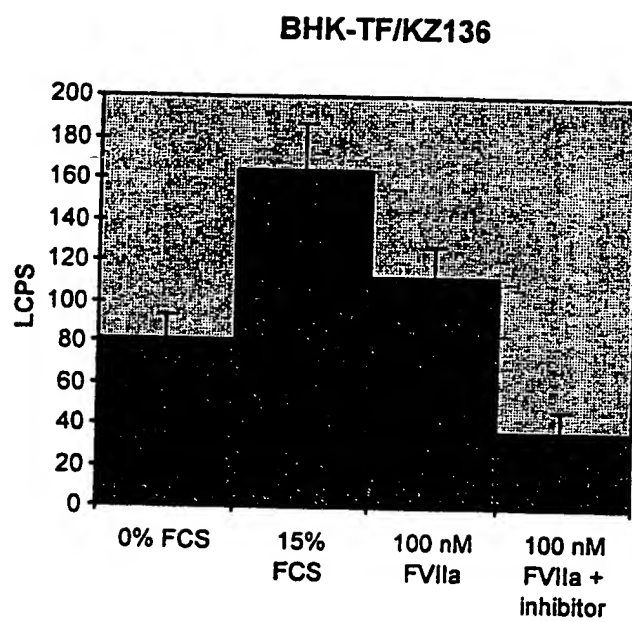


Fig. 3

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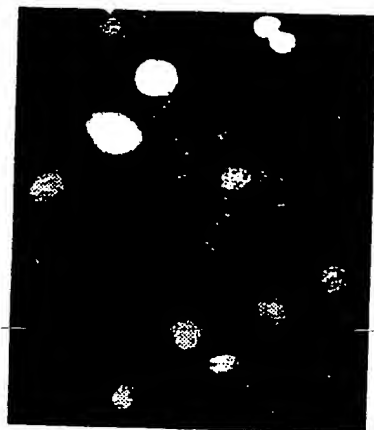


Fig. 4A

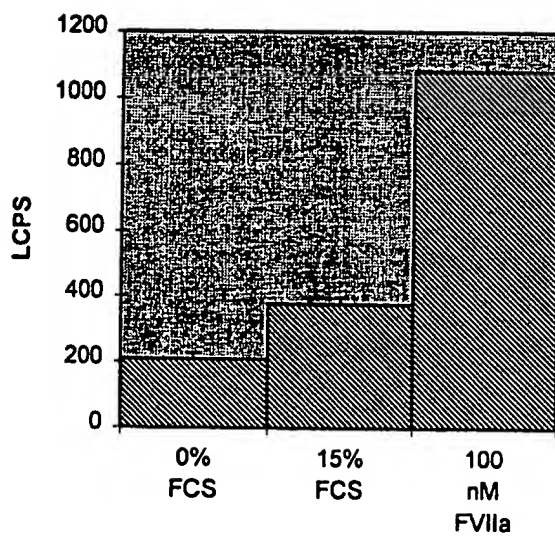


Fig. 4B

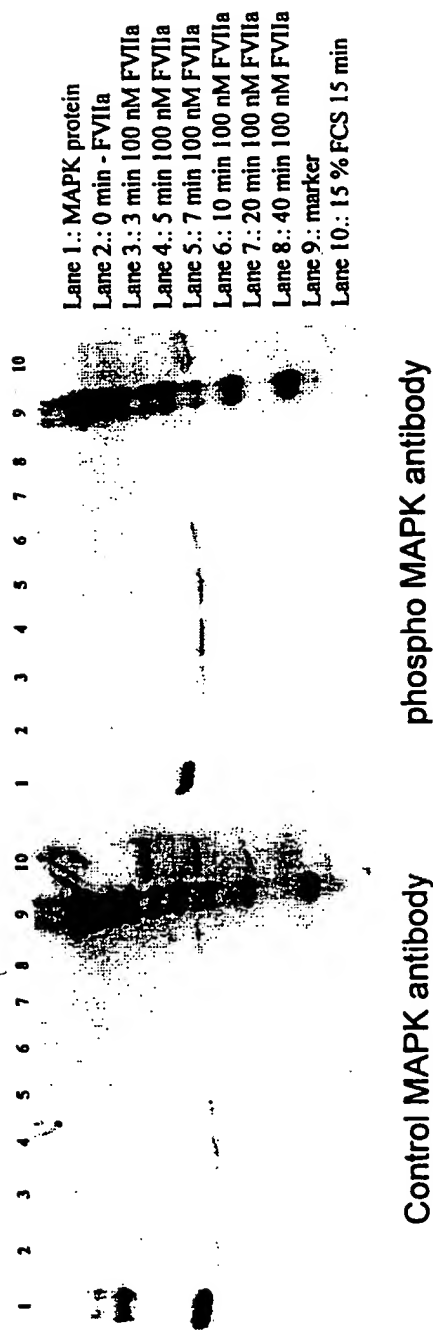


Fig. 5B

Fig. 5A

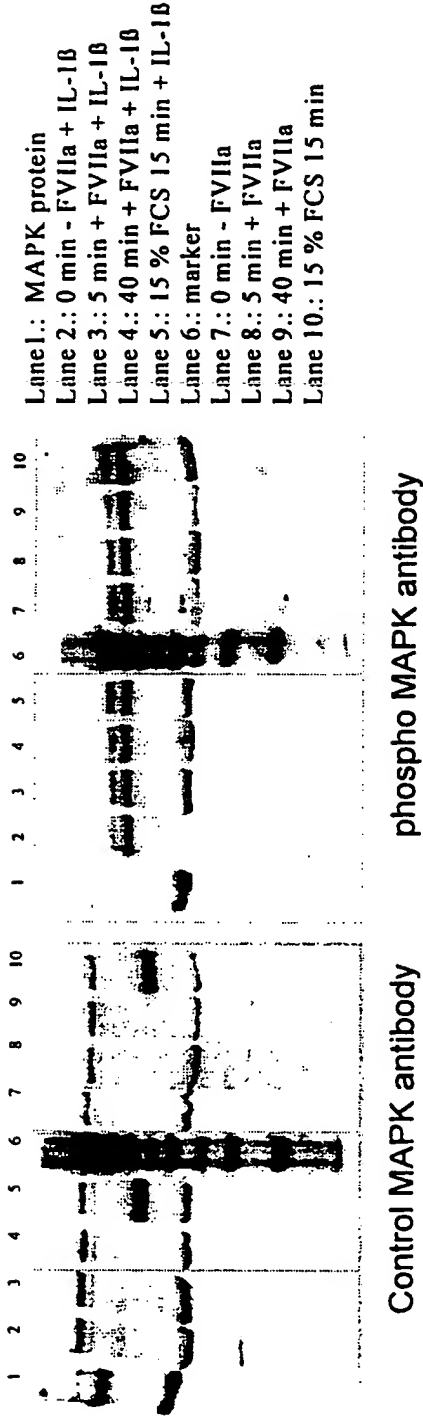


Fig. 6A

Fig. 6B

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Fig. 7B

Fig. 7A

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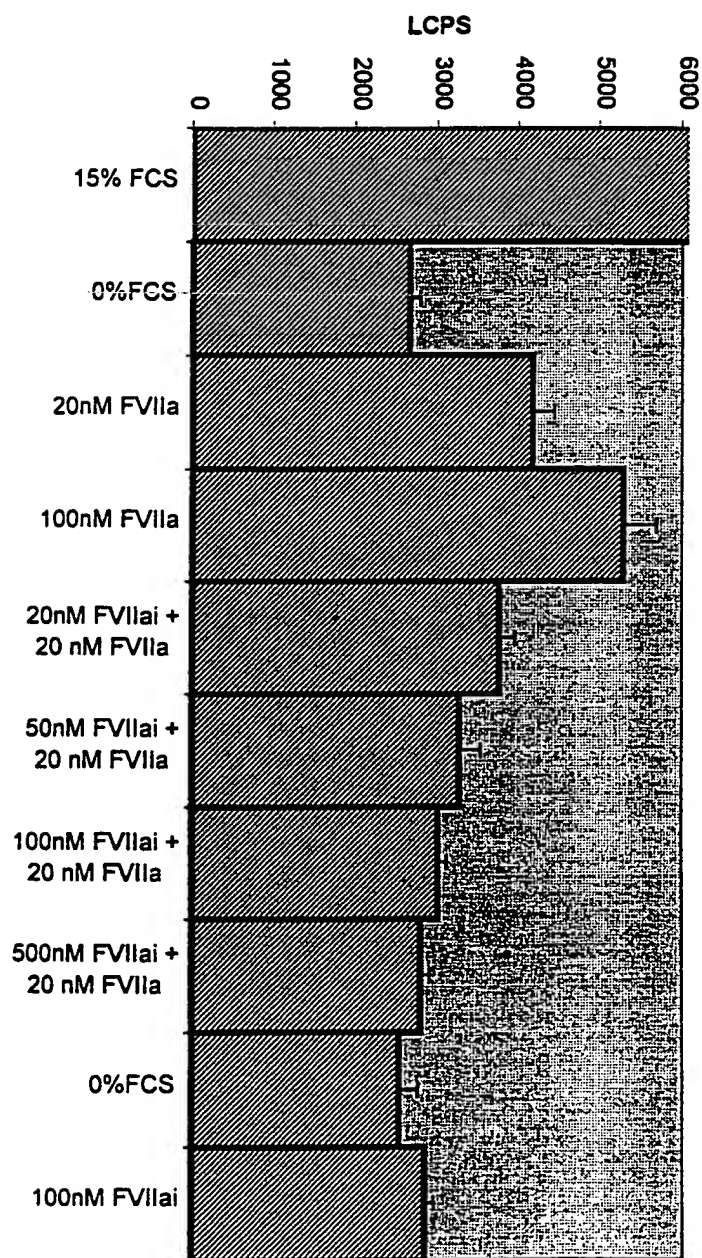


Fig. 8

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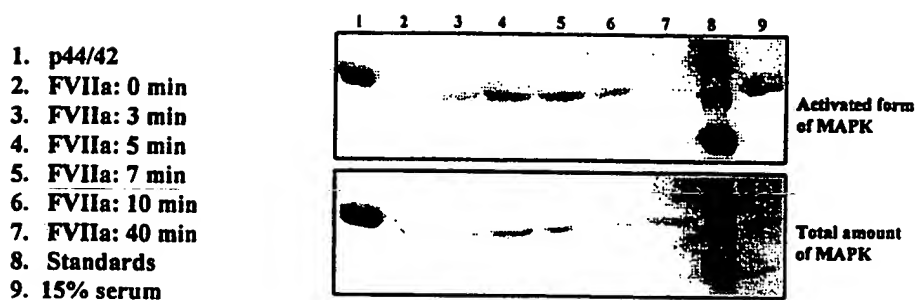


Fig. 9A

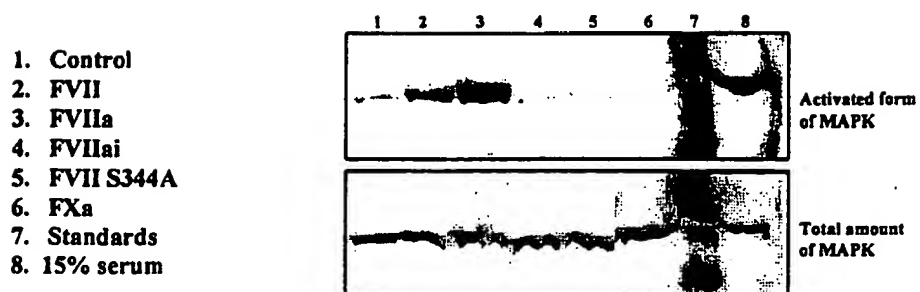


Fig. 9B

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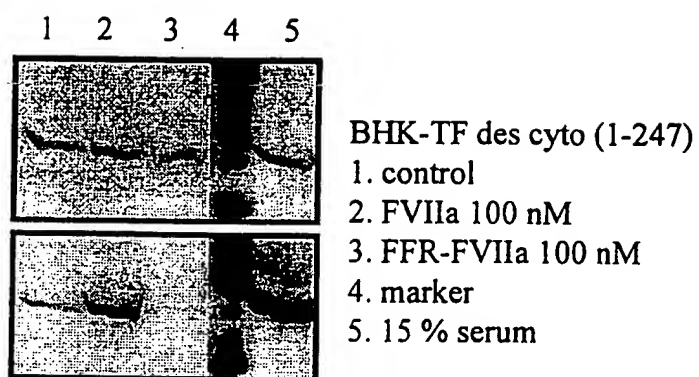


Fig. 10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK. 98/00280

A. CLASSIFICATION OF SUBJECT MATTER		
IPC6: A61K 38/48, G01N 33/53, C12Q 1/56 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC6: A61K, C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
WPI, MEDLINE, BIOSIS, EMBASE, DBA, DERWENT DRUG FILE, CA		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9612800 A1 (ZYMOGENETICS, INC. ET AL), 2 May 1996 (02.05.96), page 1, line 15 - line 18; page 4, line 5 - line 29; page 5, line 2 - line 4, page 5, line 30 --	1-14,21-32
X	EUR.J.IMMUNOL., Volume 26, 1996, Midori Masuda et al, "Association of tissue factor with an y chainhomodimer of the IgE receptor type I in cultured human monocytes", page 2529 - page 2532, the introduction and page 2530, right column, first paragraph --	1-14,16,19, 21-32
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
15 December 1998		21-12-1998
Name and mailing address of the ISA/ Swedish Patent Office B x 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer: Carolina Palmcrantz Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00280

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Dialog Information Services, File 155, MEDLINE, Dialog accession no. 09277431, Medline accession no. 97388879 (LILLE S. et al), "The regulation of p42/p44 mitogen-activated protein kinases in the injured rat carotid artery", J Surg Res (UNITED STATES) Jul 1 1997, 70 (2) p 178-86</p> <p>--</p>	1-14,16,19, 21-32
A	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 270, No 9, March 1995, John-Arne Røttingen et al, "Binding of Human Factor VIIa to Tissue Factor Induces Cytosolic Ca²⁺ Signals in J82 Cells, Transfected COS-1 Cells, Madin-Darby Canine Kidney Cells and in Human Endothelial Cells Induced to Synthesize Tissue Factor" page 4650 - page 4660</p> <p>--</p>	1-14,21-22
A	<p>Dialog Information Services, File 155, MEDLINE, Dialog accession no. 08687034, Medline accession no. 96373419, (CARMELIET P. et al), "Role of tissue factor in embryonic blood vessel development", Nature (ENGLAND) Sep 5 1996, 383 (6595) p 73-5</p> <p>--</p>	13
P,X	<p>Dialog Information Services, File 155, MEDLINE, Dialog accession no. 09499002, Medline accession no. 98200574, (OLLIVIER V. et al), "Tissue factor-dependent vascular endothelial growth factor production by human fibroblasts in response to activated factor VII", Blood (UNITED STATES) Apr 15 1998, 91 (8) p2698-703</p> <p>--</p>	1-14,16,19, 21-32

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00280

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Dialog Information Services, File 155, MEDLINE, Dialog accession no. 09462465, Medline accession no. 98165802, (POULSEN LK et al), "Signal transduction via the mitogen-activated protein kinase pathway induced by binding of coagulation factor VIIa to tissue factor", J Biol Chem (UNITED STATES) Mar 13 1998, 273 (11) p 6228-32 --	1-14,16,19, 21-32
X	Blood, Volume 86, No 2, July 1995, Carolyn L. Orthner et al, "Pyrrolidine Dithiocarbamate Abrogates Tissue Factor (TF) Expression by Endothelial Cells: Evidence Implicating Nuclear Factor-kB in TF Induction by Diverse Agonists", page 436 - page 443, the abstract --	15,18
X	WO 9405328 A1 (THE SCRIPPS RESEARCH INSTITUTE), 17 March 1994 (17.03.94), page 11, line 28 - page 12, line 3 --	15,18
A	EP 0244221 A1 (GENENTECH, INC.), 4 November 1987 (04.11.87), claims 4 and 14 --	15,18
A	WO 9640276 A1 (SUGEN, INC.), 19 December 1996 (19.12.96) --	16,19
A	WO 9722717 A1 (NOVARTIS AG), 26 June 1997 (26.06.97), page 3, line 4 - line 10 --	17,20
P,X	US 5753446 A (GARY L. JOHNSON), 19 May 1998 (19.05.98), column 4, line 6 - line 38; column 6, line 41 - line 61; column 20, line 13 - line 30 -- -----	15-20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00280

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 21-32
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 21-32 are directed to a method of treatment of the human/animal body, the search has been carried out based on the alleged effects of the compound (c.f. PCT Rule 39.1(iv)).

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next page

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00280

According to PCT Rule 13.2, an international application shall relate to one invention only or a group of inventions so linked as to form a single general inventive concept. As is stated in Annex B to Administrative Instructions under the PCT, in force July 1, 1992 (PCT GAZETTE 1992, June 25, pages 7062-9, see page 7063 and example 5) unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features-i.e. features that define a contribution which each of the inventions makes over the prior art.

A search for this special technical feature did not reveal such a unifying, novel feature among all the independent claims. Accordingly, based on the prior art which teaches that the Fviia -TF interaction results in an intracellular signal transduction, especially giving phosphorylated tyrosine residues on polypeptides (c.f. the documents cited in the search report), the following inventions were found:

Invention A, claims 1-14, 16, 19 and 21-32, pertains to the use of a TF agonist or a TF antagonist (e.g. Fvii or a modified Fviia) for the manufacture of a pharmaceutical composition for inducing/inhibiting activation of the MAPK signalling pathway in a patient.

Moreover, invention A pertains to a method of detecting a drug candidate that modulate the Fviia mediated intracellular signalling pathway, such as the MAPK signalling pathway. The method involves measuring the level of protein phosphorylation of specific proteins in the Fviia mediated intracellular signalling pathway.

Invention B, claims 15 and 18, pertains to a method of detecting a drug candidate that modulate the Fviia mediated intracellular signalling pathway, such as the MAPK signalling pathway. The method involves measuring the expression of a reporter gene during culturing of a TF expressing cell.

Invention C, claims 17 and 20, pertains to a method of detecting a drug candidate that modulate the Fviia mediated intracellular signalling pathway, such as the MAPK signalling pathway. The method involves measuring the spatial localisation of a specific component of the Fviia mediated intracellular signalling pathway that upon activation of the Fviia mediated intracellular signalling pathway change intracellular localisation.

INTERNATIONAL SEARCH REPORT
Information on patent family members

01/12/98

International application No.
PCT/DK 98/00280

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9612800 A1	02/05/96	AU 4014295 A CA 2203280 A CZ 9701228 A EP 0789759 A NO 971878 A PL 319813 A US 5788965 A US 5817788 A US 5833982 A	15/05/96 02/05/96 12/11/97 20/08/97 23/06/97 01/09/97 04/08/98 06/10/98 10/11/98
WO 9405328 A1	17/03/94	AU 5093593 A	29/03/94
EP 0244221 A1	04/11/87	SE 0244221 T3 AT 139784 T CA 1328419 A DE 3751846 D,T ES 2090007 T GR 3021017 T JP 2592063 B JP 2795833 B JP 9117285 A JP 62272990 A US 4859609 A US 5030576 A	15/07/96 12/04/94 09/01/97 16/10/96 31/12/96 19/03/97 10/09/98 06/05/97 27/11/87 22/08/89 09/07/91
WO 9640276 A1	19/12/96	AU 5965896 A EP 0873142 A	30/12/96 28/10/98
WO 9722717 A1	26/06/97	AU 7564796 A EP 0871764 A GB 9525703 D GB 9525705 D	14/07/97 21/10/98 00/00/00 00/00/00
US 5753446 A	19/05/98	AU 697340 B AU 6666394 A CA 2160548 A EP 0694044 A FI 954894 A JP 8509128 T NO 954094 A NZ 266067 A PL 311142 A US 5405941 A WO 9424159 A WO 9528421 A	01/10/98 08/11/94 27/10/94 31/01/96 13/10/95 01/10/96 13/12/95 22/09/97 05/02/96 11/04/95 27/10/94 26/10/95